

Pathogenicity and transmission studies of H5N2 parrot avian influenza virus of Mexican lineage in different poultry species

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Abstract

In 2004, a low pathogenic H5N2 influenza virus (A/parrot/CA/6032/04) was identified in a psittacine bird for the first time in the United States. Sequence and phylogenetic analysis of the hemagglutinin gene grouped the parrot isolate under the Mexican lineage H5N2 viruses (subgroup B) with highest similarity to recent chicken-origin isolates from Guatemala. Antigenic analysis further confirmed the close relatedness of the parrot isolate to Mexican lineage viruses, the highest cross-reactivity being demonstrated to Guatemala isolates. In vivo studies of the parrot isolate in chickens, ducks and turkeys showed that the virus, though did not cause any clinical signs, could replicate to high titers in these birds and efficiently transmit to contact control cage mates. The possibility that the parrot harboring the virus was introduced into the United States as a result of illegal trade across the border provides additional concern for the movement of foreign animal diseases from neighboring countries. Considering the potential threat of the virus to domestic poultry, efforts should be continued to prevent the entry and spread of influenza viruses by imposing effective surveillance and monitoring measures.

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1. Introduction

Avian influenza (AI) is caused by viruses of the family Orthomyxoviridae. These viruses are segmen-

ted, single stranded RNA viruses. They have been divided into types A, B and C based on the antigenic properties of two of their internal proteins, the matrix protein and nucleoprotein (Webster et al., 1992). Type A, which is the only type that causes natural infection in birds (Alexander, 2000) has been divided into sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes based on serologic tests (Hinshaw et al., 1982; Kawaoka et al., 1990; Rohm et al., 1996;

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Fouchier et al., 2005). Avian influenza viruses in general are classified into two pathotypes, low pathogenic (LP) and high pathogenic (HP) AI. The HPAI forms have only been identified in the H5 and H7 subtypes of the virus (Swayne and Suarez, 2000) and are of grave concern to poultry and animal populations.

Recently, a LPAI subtype H5N2, A/parrot/California/6032/04 (PS/CA/04) virus was isolated from a red-lored amazon parrot (*Amazona autumnalis autumnalis*) in California, United States (U.S.) with symptoms of lethargy, diarrhea and dehydration (Hawkins et al., 2006). It was the first report of a H5N2 virus of Mexican lineage in the U.S. and the first one in a psittacine bird. It was speculated that the virus might have been introduced into the U.S. by illegal bird trade from Mexico or Central America. Since their initial isolation from chickens in 1994, Mexican lineage H5N2 viruses continue to circulate in Mexico and spread to adjacent countries such as Guatemala and El-Salvador despite massive vaccination programs (Wright et al., 2006). Recent studies showed that the Mexican lineage AI viruses were undergoing antigenic drift away from the vaccine strain and current inactivated vaccines were ineffective in preventing viral shedding in chickens challenged with recent field isolates (Lee et al., 2004). In 2005, Japan reported an LPAI H5N2 outbreak and the causative virus was found to be most closely related to the virus isolated from Guatemala (Ozawa et al., 2006).

Little information is available on the potential role of psittacine birds on the ecology, amplification, pathogenicity and transmission of AI viruses among domestic birds (Perkins and Swayne, 2003). In 1997 and 1998, H9N2 influenza A viruses were isolated from Indian ring-necked parakeets whose HA and NA genes showed greater than 97% sequence similarity to H9N2 viruses isolated from humans in Hong-Kong (Mase et al., 2001). Psittacine birds have also been implicated in the transmission of Newcastle disease virus in California in 1970s (Utterback and Schwartz, 1973) and are speculated to act as biological vectors for avian influenza viruses (Mase et al., 2001). Hence, the recent parrot origin H5N2 virus raises significant questions concerning their impact on poultry and human health.

In order to assess the potential threat of this parrot isolate for domestic poultry, we conducted

pathogenicity and transmission studies with this virus in chickens, ducks and turkeys.

2. Materials and methods

2.1. Virus

The viruses used in this study (Fig. 1 and Table 1) were obtained from the repository of Southeast Poultry Research Laboratory (SEPRL), Athens, GA. The viruses were passaged once or twice in 10-day-old embryonated chicken eggs (ECE) to make working stocks of the virus.

2.2. Sequencing and phylogenetic analysis

RNA extraction and RT-PCR amplification of HA genes of A/chicken/Guatemala/270475-1/03 (CK/Guate/03) and A/chicken/Guatemala/270475-4/03 isolates were done as previously described (Lee et al., 2004). Direct sequencing was performed with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PerkinElmer, Foster City, CA) run on a 3730 automated sequencer (PerkinElmer). Sequence comparisons to selected viruses were conducted by using the Megalign program using the Clustal V alignment algorithm (DNASTAR, Madison, WI), and phylogenetic relationships were estimated by the method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc., Sunderland, MA) using a bootstrap resampling method with a heuristic search algorithm. The HA sequences of Guatemala isolates have been deposited in the GenBank database (accession nos. EU099400 and EU099401). The sequences of the parrot isolate (PS/CA/04) and other Mexican isolates have been reported previously (Lee et al., 2004; Hawkins et al., 2006).

2.3. Hyperimmune sera production and hemagglutination inhibition (HI) test

For raising antisera, viruses were grown in 10-day-old ECE, the infectious pooled allantoic fluid was inactivated with 0.1% beta-propiolactone (Sigma, St. Louis, MO) and the inactivated virus was used to prepare an oil-emulsion vaccine as

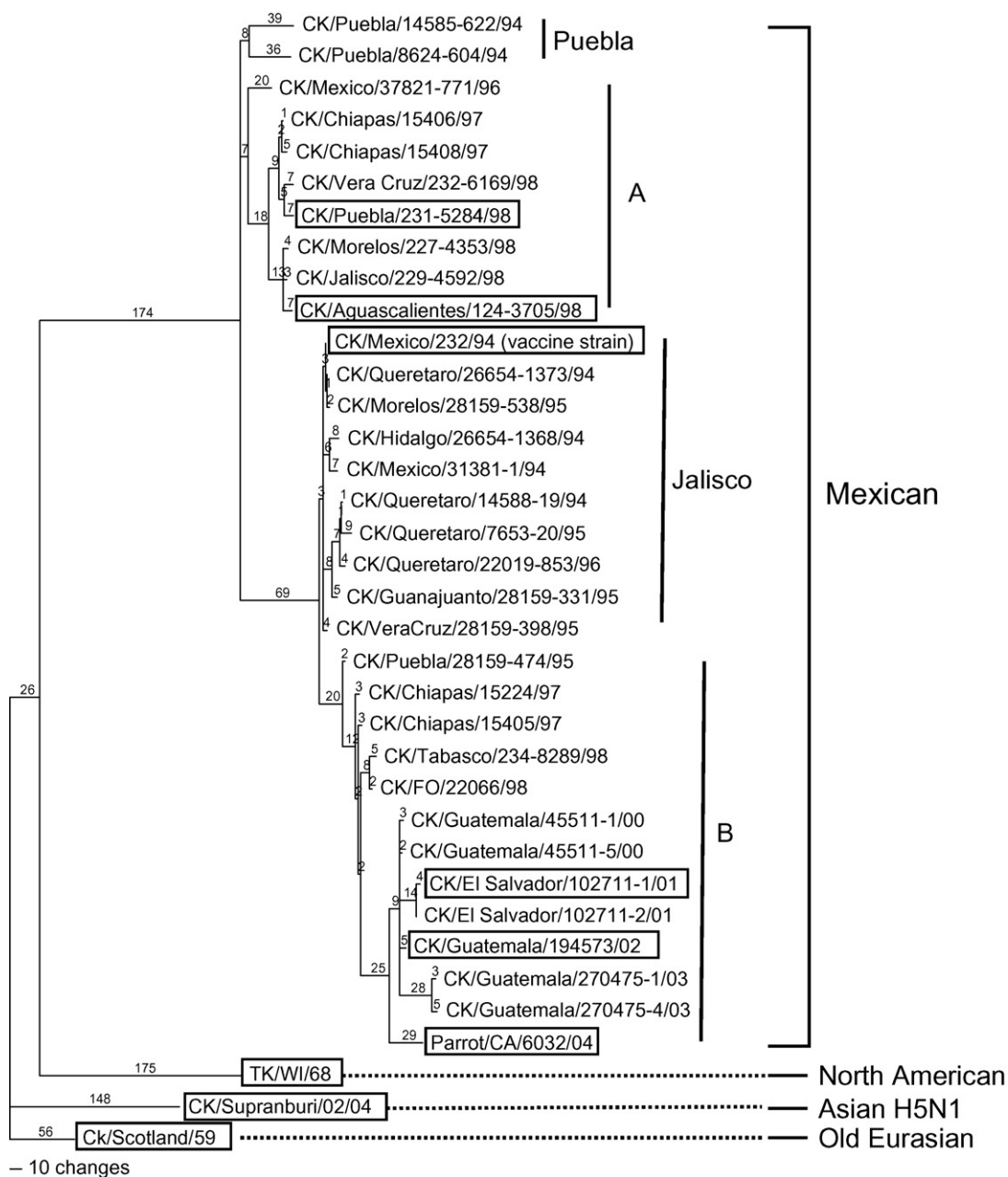


Fig. 1. Phylogenetic tree based on nucleotide sequence of the HA1 genes from H5 isolates. The tree was generated by the maximum parsimony method with the PAUP4/0b10 program with bootstrap replication (1000 bootstraps) and a heuristic search method. The tree is rooted to CK/Scotland/59, and branch lengths (which indicate number of nucleotide sequence difference) are included on the tree. Isolates used in antigenic analysis are indicated by box. Abbreviations: CK, chicken; TK, turkey. Standard two-letter abbreviations are used for states in the United States.

Table 1
Antigenic relatedness determined by hemagglutination inhibition (HI) test

Antigen	Antiserum									
	Mex. vacc	CK/Pu/98	CK/ES/01	CK/Gu/02	CK/Gu/03	PS/CA/04	TK/WI/68	CK/Sct/59	CK/Th/04	CK-H7N2
Homologous	1024 ^a	1024	1024	1024	1024	1024	1024	1024	1024	1024
CK/Guate/03	128	256	512	1024	1024	1024	32	256	20	<4
PS/CA/04	64	256	256	512	512	1024	32	256	10	<4

Abbreviations used: Mex Vacc: CK/Mexico/232/94 (vaccine strain), CK/Pu/98: CK/Puebla/231-5284/98, CK/ES/01: CK/El-Salvador/102711-1/01, CK/Gu/02: CK/Guatemala/194573/02, CK/Gu/03: CK/Guatemala/270475/03, PS/CA/04: Parrot/California/6032/04, TK/WI/68: Turkey/Wisconsin/68, CK/Sct/59: CK/Scotland/59, CK/Th/04: CK/Supranburi/02/04, CK-H7N2: CK/NJ/150383-7/02.

^a The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

previously described (Stone et al., 1997). The vaccine was inoculated subcutaneously into 3-week-old specific pathogen-free (SPF) chickens. The birds were bled on the 3rd week after vaccination to determine the HI titer of the sera and booster vaccinated in the same way with the same amount of inoculum. Two weeks after booster vaccination, the birds were bled and the serum tested for antibodies utilizing the HI test.

The HI assay was performed as described (Beard, 1989). Four hemagglutinating units of antigen (homologous and heterologous viruses) was added to a total volume of 25 μ l per well in a 96-well plate. An equal volume of serially diluted serum was added to each well and incubated for 30 min after which an equal volume of 0.5% (v/v) chicken erythrocytes was added to each well. The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

2.4. Pathogenicity and replication of PS/CA/04 and CK/Guate/03 isolates in chickens

Two groups of 3-week-old SPF chickens were inoculated intranasally with 0.2 ml allantoic fluid containing 10^5 50% egg infectious dose (EID₅₀)/0.2 ml of PS/CA/04 or CK/Guate/03 viruses, respectively. Tracheal and cloacal swabs were collected from the infected birds at 3 and 5 days post-infection (dpi). The swabs were suspended in 2 ml of brain-heart infusion broth with antibiotics. Serially diluted samples were injected into 10-day-old ECE for virus titration (Reed and Muench, 1938). Serum was collected at 2 weeks post-infection and HI titers were determined as described above.

2.5. Comparative pathogenicity, replication and transmission of PS/CA/04 isolate in chickens, ducks and turkeys

Four-week-old SPF chickens (Charles River Laboratories, Inc. Wilmington, MA), 2-week-old commercial Pekin ducks (Ridgeway Hatcheries, Inc. LaRue, OH) and 3-week-old Egglime turkeys (OARDC flock) were used in the present study. Eleven birds of each species were inoculated with 0.2 ml of 10^6 EID₅₀ of the virus through intratracheal route. Four birds were introduced as contact controls at 1 dpi. Tracheal and cloacal swabs were collected from all the birds on days 2, 4 and 7 post-infection (days 1, 3, 6 for contact controls) and also on day 14 (day 13 for contact control) from ducks and turkeys. Individual swabs were placed in 1.5 ml of sterile phosphate buffered saline (PBS) containing gentamycin (1 mg per 100 ml). RNA was extracted from the tracheal and cloacal swabs with RNeasy Minikit (Qiagen, Inc., Valencia, CA) in accordance with the manufacturer's instructions. The virus was quantitated by real-time RT-PCR (RRT-PCR) directed to the matrix (M) gene as previously reported (Lee and Suarez, 2004). To prepare control RNA that can be used to generate standard curve, we extracted RNA from serially diluted allantoic fluid that contains known titer of the PS/CA/04 virus (10^3 to 10^7 EID₅₀/0.2 ml). The average Ct values for each viral dilution were 20.37, 23.8, 27.44, 30.73 and 34.65, respectively, with co-efficient of correlation values (r^2) of > 0.98. For quantitation, swab samples were run together with these control RNA and the amount of RNA in the samples was converted into EID₅₀/0.2 ml by interpolation.

At 3 dpi, three infected chickens and four infected turkeys and ducks were euthanized and tissues (trachea, lungs, kidney, bursa, cloaca, spleen, portions of small and large intestine, cecal tonsils) were collected for histopathology. All the infected birds were bled for serum collection on days 7 and 14 post-infection (days 6 and 13 for contact controls) but only the ducks and chickens were sampled at day 21 post-infection (day 20 for contact controls). The HI tests were performed with 1% turkey or duck erythrocytes as described above.

2.6. Histopathology and immunohistochemistry (IHC)

Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μ m and were stained with hematoxylin and eosin (HE). A duplicate 4- μ m section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein, developed at Southeast Poultry Research Laboratory, was used as the primary antibody for a streptavidin–biotin–alkaline phosphatase complex-based IHC method as previously described (Swayne, 1997).

3. Results

3.1. Phylogenetic and antigenic analysis

In this study, we sequenced the HA genes of chicken viruses isolated from Guatemala in 2003. Both isolates had almost identical sequence and shared high similarity with the parrot (PS/CA/04) and other Mexican lineage viruses. Mexican lineage viruses have been classified previously into four different clusters; the ones isolated before 1996 were separated into the Jalisco and Puebla sublineages and viruses isolated after 1995 were separated into the sublineages A and B which include viruses isolated from Guatemala and El-Salvador (Lee et al., 2004). The parrot isolate formed a cluster with Guatemala and El-Salvador isolates under sublineage B and shared 96–98% HA1 gene nucleotide

similarity. Phylogenetically, Mexican lineage viruses, including the parrot isolate, were clearly separated from other Eurasian and North American lineage H5 viruses (Fig. 1).

We conducted HI tests to compare the antigenicity of PS/CA/04 with H5 viruses of Mexican lineage, North American lineage (A/turkey/Wisconsin/68), Eurasian lineage (A/chicken/Scotland/59) and Asian H5N1 (A/chicken/Supranburi/02/04) viruses. Antibody raised against H7N2 subtype virus, A/turkey/New York/4450/94, was included as a negative control. The sera raised against the parrot isolate showed high cross-reactivity with the Guatemala isolates, showing that the viruses are antigenically closely related to each other (Table 1). Similarly, antisera against Guatemala isolates showed only twofold difference in the HI titer with the parrot isolate confirming the antigenic similarity of these two viruses. The Guatemala and parrot isolates showed 4- to 16-fold differences in HI titers with the Mexican lineage viruses including the vaccine strain. The parrot isolate was antigenically distinct from North American and Asian H5N1 viruses and 32- and 64-fold differences in HI titers were observed. No cross-reactivity (< 4 HI titer) was observed with H7 subtype virus.

3.2. Replication of the PS/CA/04 and CK/Guate/03 isolates in chickens

In vivo characterization of CK/Guate/03 and PS/CA/04 with a 5.0 log₁₀ EID₅₀ inoculation showed similar tissue tropism and replication patterns for both viruses (Table 2). The mean virus titer in the tracheal swabs of infected chickens at 3 dpi were found to be 5.5 and 5.2 log₁₀ EID₅₀/0.2 ml of swab fluid for CK/Guate/03 and PS/CA/04, respectively, whereas no virus was detected from the cloacal swab samples in both cases. At 5 dpi, the mean tracheal titers were the same (4.0 log₁₀ EID₅₀/0.2 ml) for both groups of birds. Only one bird infected with CK/Guate/03 showed a positive cloacal titer of 3.68 log₁₀ EID₅₀/0.2 ml at 5 dpi and no other swab samples contained the detectable RNA. The mean HI titer at 14 dpi was found to be 8.4 and 7.9 log₂ for birds infected with CK/Guate/03 and PS/CA/04, respectively. No clinical signs were observed in any of the infected birds during the 14-day observation period.

Table 2

Replication of A/chicken/Guatemala/270475/03 and A/parrot/California/6032/04 isolates in chickens

Virus	Swabs	Virus isolation		HI titer ^c 14 dpi ^d
		3 dpi	5 dpi	
CK/Guatemala/270475/03	Tracheal	5.5 + 0.3 ^a (4/4) ^b	4.0 + 0.8 (4/4)	8.4 + 1.4
	Cloacal	0.0 + 0.0 (0/4)	3.7 + 0.0 (1/4)	
PS/California/6032/04	Tracheal	5.2 + 0.3 (4/4)	4.0 + 0.2 (4/4)	7.9 + 0.6
	Cloacal	0.0 + 0.0 (0/4)	0.0 + 0.0 (0/4)	

^a log₁₀ 50% egg infectious dose (EID₅₀)/0.2 ml of swab fluid + standard deviation.^b Number of positives/total numbers tested.^c Log₂ hemagglutination inhibition (HI) titer of the antisera + standard deviation.^d Days post-infection.

3.3. Pathogenicity and transmission studies of PS/CA/04 virus in different poultry species

Comparative pathogenicity and transmission studies in chickens, ducks and turkeys were undertaken with the parrot H5N2 isolate by infecting birds with 10^{6.0} EID₅₀ of the virus. None of the infected or contact control birds of the three species tested showed any clinical signs of disease or fatality after the virus inoculation. In infected chickens, mean virus titers of 4.7, 4.6 and 3.3 log₁₀ EID₅₀/0.2 ml were detected from the tracheal swabs on days 2, 4 and 7 dpi (Table 3). The tracheal swabs from all except one contact control chicken were positive with mean titers

of 3.6, 5.0 and 4.8 log₁₀ EID₅₀/0.2 ml on days 1, 3 and 6 post-infection. The cloacal swabs were negative for most of the birds on the days tested except for one infected bird on 2 dpi and a contact control bird on 7 dpi. All the experimentally inoculated chickens and the contact control birds sero-converted. The HI titer ranged from 1.0 to 8.3 log₂ showing an increase from 7 to 21 dpi (Table 3).

In infected ducks, the mean viral titers in the trachea were lower (4.0, 1.9, 0.3 and 0.9 log₁₀ EID₅₀/0.2 ml of swab fluid) on days 2, 4, 7 and 14 dpi compared to infected chickens (Table 3). None of the ducks showed positive cloacal swabs on 2 dpi, but on days 4 and 7, a mean cloacal swab titer of

Table 3

Pathogenicity and transmission study of A/parrot/California/6032/04 in chickens, ducks and turkeys

Species	Group	Swabs	Virus isolation ^a				HI titer ^b		
			2 dpi ^c	4 dpi	7 dpi	14 dpi	7 dpi	14 dpi	21 dpi
Chicken	Infected	Tracheal	4.7 + 0.5 (11/11) ^d	4.6 + 0.8 (9/9)	3.3 + 1.1 (9/9)	ns	4.8 + 0.7	6.9 + 0.8	8.3 + 0.5
		Cloacal	0.2 + 0.6 (1/4)	0 (0/4)	0 (0/4)				
	Contact control	Tracheal	3.6 + 0.2 (3/4)	5.0 + 0.4 (4/4)	4.8 + 0.4 (4/4)	ns	1.0 + 0.8	6.5 + 1.3	7.5 + 1.3
		Cloacal	0 (0/4)	0 (0/4)	0.4 + 0.9 (1/4)				
Ducks	Infected	Tracheal	4.0 + 1.3 (8/11)	1.9 + 1.0 (6/7)	0.3 + 0.7 (1/7)	0.9 + 1.7 (2/7)	3.7 + 1.3	5.2 + 0.8	5.3 + 1.5
		Cloacal	0 (0/7)	1.6 + 2.3 (3/7)	1.6 + 1.8 (4/7)	0 (0/7)			
	Contact control	Tracheal	0 (0/4)	2.9 + 0.5 (4/4)	3.1 + 2.3 (3/4)	0 (0/4)	1.0 + 1.2	1.8 + 0.5	3.0 + 0.0
		Cloacal	0 (0/4)	0 (0/4)	4.3 + 0.5 (4/4)	0.6 + 0.7 (2/4)			
Turkeys	Infected	Tracheal	1.7 + 1.5 (8/11)	4.0 + 0.5 (7/7)	2.2 + 0.8 (7/7)	0 (0/7)	4.2 + 1.2	8.0 + 1.4	ns
		Cloacal	0.6 + 0.9 (4/11)	2.0 + 1.6 (5/7)	2.4 + 1.7 (5/7)	0.5 + 1.4 (1/7)			
	Contact control	Tracheal	2.0 + 1.4 (3/4)	3.7 + 0.8 (3/4)	2.8 + 0.7 (3/3)	0 (0/4)	3.0 + 0.8	7.3 + 1.9	ns
		Cloacal	1.1 + 0.8 (3/4)	0.7 + 1.5 (1/4)	1.8 + 1.2 (1/4)	0 (0/4)			

ns: not sampled.

^a Log₁₀ 50% egg infectious dose (EID₅₀)/0.2 ml of swab fluid + standard deviation determined by RRT-PCR.^b Log₂ hemagglutination inhibition (HI) titer of the antisera + standard deviation.^c Days post-infection.^d Number of positives/total numbers tested.

1.6 log₁₀ EID₅₀/0.2 ml of swab fluid was observed in 3 and 4 birds, respectively. Virus could also be demonstrated in the tracheal and cloacal swabs of contact control birds. All of the infected and contact control ducks sero-converted. The mean HI titers in infected and contact control ducks ranged from 3.7 to 5.3 log₂ and 1.0 to 3.0 log₂, respectively, from 7 to 14 dpi.

The mean tracheal and cloacal swab titers in turkeys ranged from 1.7 to 4.0 and 0.6 to 2.4 log₁₀ EID₅₀/0.2 ml, respectively, from 2 to 7 dpi (Table 3). The virus was efficiently transmitted to the contact control birds as shown by the positive viral titers in tracheal and cloacal swabs. In infected turkeys, the mean HI titers ranged from 4.2 to 8.0 log₂ and in contact control turkeys from 3.0 to 7.3 log₂ at 7 and 14 dpi, respectively.

Histopathologically, in all three species, focal mild lymphohistiocytic interstitial pneumonia with mild diffuse pulmonary edema and congestion were observed. Minimal viral antigen staining (less than 3 positive cells per high-power field (400×)) could only be observed in infiltrating macrophages. Mild to moderate bronchitis was present in some birds with bronchial epithelial hyperplasia, deciliation and epithelial degeneration. Mild staining (> 3 and < 10 positive cells per high-power field) for viral antigen was present in bronchial epithelial cells (Fig. 2). Tracheas from chickens lacked lesions, however some presented deciliation and mild lymphocytic infiltration in the submucosa. In turkeys and ducks, mild to severe lymphoplasmacytic tracheitis was observed (Fig. 2). The tracheal mucosa had diffuse epithelial cell hyperplasia and focal

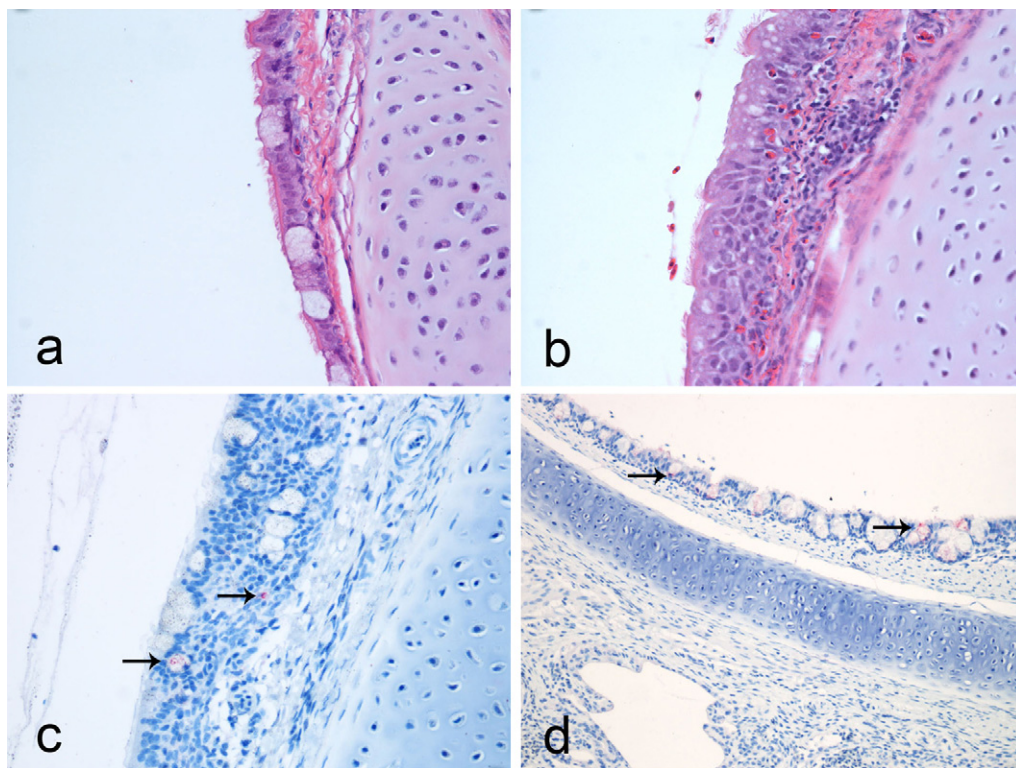


Fig. 2. Histological lesions in turkeys (a, b, and d) and ducks (c) infected with PS/CA/04 H5N2 avian influenza virus. Photomicrographs of hematoxylin and eosin stained tissue sections (a and b) and sections stained by IHC methods to demonstrate viral antigens (c and d). (a) Trachea from a normal turkey (400×), (b) trachea from a virus inoculated turkey: severe lymphoplasmacytic tracheitis (400×), (c) trachea from a virus inoculated duck: viral antigen staining in epithelial cells and infiltrating macrophages (arrows) (400×), (d) secondary bronchus from a virus inoculated turkey: viral antigen staining in bronchial epithelial cells (arrows) (400×).

deciliation, and the submucosa was diffusely infiltrated with lymphocytes, plasma cells, macrophages, and in some cases heterophils (more commonly seen in turkeys). Occasionally, exudates with fibrin and cellular debris were present within the lumen. Staining for viral antigen was rarely present in epithelial cells, infiltrating macrophages, and in desquamated epithelial cells (Fig. 2). Other microscopic lesions observed were mild atrophy of lymphoid follicles in the bursa and increased numbers of lymphoid follicles in cecal tonsils. Less commonly observed were increased lymphocytes in the intestinal submucosa and some desquamation of intestinal epithelium, and lymphocytic infiltration in kidneys.

4. Discussion

Influenza viruses have rarely been isolated from psittacine birds and little information is available on the pathogenicity of these viruses in psittacine birds (Mase et al., 2001; Perkins and Swayne, 2003; Hawkins et al., 2006). Their isolation from psittacine birds has usually been confined to quarantine locations and they have not been implicated as reservoirs of influenza viruses (Mase et al., 2001). However, the isolation of H9N2 viruses from parakeets and their close relation to the viruses isolated from affected people in Hong-Kong imply that influenza viruses in these birds can be a potential threat to human and animal health. Hence, the recent isolation of an LPAI H5N2 virus of Mexican lineage from a parrot in the U.S. raises questions concerning its potential pathogenicity and transmission among domestic birds.

Phylogenetic and antigenic analysis grouped the parrot virus with other viruses of Mexican lineage within the subgroup B showing highest relatedness to Guatemala isolates (Fig. 1 and Table 1). This high genetic similarity of parrot isolate to Mexican lineage viruses has been reported earlier by Hawkins et al. (2006). Also, experimental infection in chickens with the parrot and CK/Guate/03 isolates showed that the two viruses have similar biological characteristics (Table 2). Both viruses replicated well in the upper respiratory tract, but had no affinity to the digestive tract of chickens. This similarity in biological characteristics and genetic and phylogenetic studies suggested that the parrot isolate virus might be poultry

adapted virus and could easily replicate and transmit among domestic birds.

To further assess the pathogenic potential of the parrot isolate to domestic poultry, we undertook pathogenicity and transmission studies in chickens, ducks and turkeys. Limited lesions and AI viral antigen in the trachea and bronchi of chickens infected with the parrot isolates are typical of LPAI where infection is limited to the respiratory tract in general (Mo et al., 1997; Swayne, 1997). The more severe lesions or clinical signs observed in the reported case in the Amazon parrot and severe disease reported with other LPAI viruses suggests secondary bacterial or other viral pathogens in the field that may induce a more severe disease than produced by the LPAI virus alone.

All the experimentally infected and contact control chickens showed a positive viral titer in the tracheal swabs whereas the cloacal swabs were negative for most birds, except an infected bird at 2 dpi and a contact control bird at 7 dpi. In ducks and turkeys, the virus was demonstrated in both cloacal and tracheal swabs, but the viral titers in the tracheal swabs were lower compared to chickens. These results showed that even though the virus could propagate and transmit efficiently in these three species of birds, there is a distinct species difference in biological characteristics of the virus. In chickens, high viral titers were observed from the tracheal swabs at 2 and 4 dpi and the titers slowly declined at 7 dpi. However in ducks, high tracheal titers were observed at 2 dpi, whereas by 4 dpi, the titer had declined steeply and was almost negligible at 7 dpi. In turkeys, the titers increased from 2 to 4 dpi and declined by 7 dpi. Chickens demonstrated better and prolonged replication of the virus in the upper respiratory tract, ducks allowed good initial replication, but sustained it for a shorter time, whereas in turkeys, the replication slowly increased from 2 to 4 dpi and then declined. In chickens and turkeys, the tracheal titers were consistently higher than the cloacal titers for all 3 days examined, whereas for ducks, the cloacal titers were higher for the infected and contact control birds at 4 and 7 dpi. This is in accordance with previous studies that reported the intestinal tract of ducks to support the virus better than the respiratory tract (Webster et al., 1992; Sturm-Ramirez et al., 2005; Morgan, 2006). Hence, the fecal–oral route is believed

to be the common mode of transmission in ducks. The exact mechanism of pathogenesis and tissue tropism of avian influenza virus is unknown, and it is believed to be multi-factorial (Sweet and Smith, 1980) and dependant on the virus strain, host, host age, availability and distribution of receptors and management conditions. The sialic acid type in different organs and the pH stability of neuraminidase are known to play a role in determining the tissue tropism of different viruses (Suzuki, 2005; Wan and Perez, 2006). Even though previous reports indicate an abundance of sialic acid α 2,3-galactose-linked receptors in chicken trachea and intestine (Wan and Perez, 2006), the reason for the preferential multiplication of the virus in the respiratory tract of chicken is unknown. Further detailed studies are required to elucidate the receptor profile and tissue tropism of influenza viruses in different poultry species.

Serological results revealed that all the infected and contact control birds sero-converted as shown by the high HI titers among the three species of birds. This suggests efficient horizontal transmission of the virus to the contact control birds. The HI titers were found to be the lowest in ducks among the three species tested. To rule out any inconsistent results as previously reported (Brown et al., 2006), we performed HI test with chicken, duck and turkey erythrocytes and the results were found to be consistent (data not shown). The low immunological response in ducks could be due to the adaptation of the virus in these hosts over a long period of time, resulting in a reservoir that propagates the virus without any adverse effects on the host. However, the 1997–2000 H7N1 influenza outbreaks in Italy and the 2001 H5N1 Hong-Kong outbreak wherein aquatic birds were affected raises question about this concept of evolutionary stasis and maintenance of equilibrium (Sturm-Ramirez et al., 2005; Chen and Holmes, 2006). Detailed understanding of the pathological and immunological mechanisms of influenza virus in different bird species is necessary to explain the results observed.

Most of the influenza viruses reported to be isolated from psittacine birds are usually from birds held in quarantine after their importation (Mase et al., 2001). It is speculated that the parrot from which the virus was isolated might have been introduced into the U.S. as a result of illegal bird trade across the border. The replication and transmission studies with this parrot

isolate show that the virus can effectively infect domestic birds. Also, it is well known that persistence of the virus and continuous field passage can mutate an LPAI to HPAI virus and even result in changes that allow a virus to more easily cross the species barrier to infect mammals or human beings. This warrants effective surveillance of domestic birds in the U.S. and effective monitoring at the borders to prevent illegal import of birds harboring disease agents.

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